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REPORTS

Enhanced Cancer Growth in Mice Administered Daily Human-Equivalent Doses of Some H_1-Antihistamines: Predictive In Vitro Correlates

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Background: Present studies of drug-induced tumor growth promotion have evolved from earlier investigations into the mechanism of action of *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine · HCl, a tamoxifen derivative which potently inhibits lymphocyte mitogenesis in vitro and stimulates tumor growth in vivo. It is thought that potency to bind to intracellular histamine receptors (H_{1C}), some of which are on cytochromes P450, may correlate with tumor growth-promoting activity. ***Purpose:*** We assessed the effectiveness of five in vitro assays in predicting in vivo tumor growth stimulation by the H_1-antihistamines loratadine, astemizole, cetirizine, hydroxyzine, and doxylamine. ***Methods:*** Potency of each agent was ranked 1-5 in each of the following in vitro assays: 1) inhibition of [^{3}H]histamine binding to microsomal H_{1C}, 2) inhibition of histamine binding to microsomal P450, 3) inhibition of the P450-catalyzed demethylation of aminopyrine, 4) inhibition of lymphocyte mitogenesis, and 5) stimulation of tumor colony formation. An overall rank score was assigned to each drug and correlated with tumor growth stimulation in vivo. Two laboratories conducted in vivo studies in a blinded fashion. Female C57BL and C3H mice were given a subcutaneous injection on day 1 of syngeneic B16F10 melanoma cells (5×10^5) or C-3 fibrosarcoma cells (1×10^5), respectively. Mice were randomly assigned to treatment groups, then received a single, daily intraperitoneal injection of an estimated human-equivalent dose (or range of doses) of antihistamine or vehicle control for 18-21 days before being killed. Tumors were surgically removed and wet weights compared statistically among groups. ***Results:*** The cumulative potency of each drug in affecting tumor growth or growth mechanisms in the five in vitro assays ranked as follows: Loratidine and astemizole ranked highest and were equally potent, followed in decreasing order by hydroxyzine, doxylamine, and cetirizine. A significant correlation (r = .97; P<.02) was observed between the rank order of potency of the antihistamines in all five in vitro assays and the rank order to enhance tumor growth in vivo: Loratidine and astemizole significantly (P<.001) promoted the growth of both melanoma and fibrosarcoma, hydroxyzine significantly (P<.001) promoted the growth of melanoma, while doxylamine and cetirizine did not promote the growth of either tumor. ***Conclusion:*** Data demonstrate that the in vitro assays predicted the propensity of each H_1-antihistamine to stimulate cancer growth in vivo. ***Implication:*** These in vitro tests may prove valuable to screen potential tumor growth promoters. [J Natl Cancer Inst 86:770-775, 1994]

We (*1*) reported that, at human-equivalent doses, two nongenotoxic antidepressant drugs, amitriptyline (Elavil, Stuart Pharmaceuticals, Wilmington, Del.) and fluoxetine (Prozac, Dista Division, Eli Lilly and Co., Indianapolis, Ind.), accelerate the growth of cancer in rodents. Subsequently, others (*2,3*) confirmed a similar property for clomipramine (Anafranil, Basel Pharmaceuticals, Summit, N.J.) and desipramine (Norpramin, Marion Merrell Dow Inc., Kansas City, Mo.), both tricyclic analogues of amitriptyline. Our studies of drug-induced tumor growth promotion arose from earlier investigations into the mechanism of action of *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine · HCl (DPPE), a tamoxifen derivative that potently inhibits lymphocyte mitogenesis in vitro (*4*) and stimulates tumor growth in vivo (*5*). Through binding to microsomal and nuclear anti-estrogen-binding sites (AEBS) (*6*), DPPE inhibits the binding of [^{3}H]histamine to AEBS-associated intracellular histamine receptors (H_{1C}) implicated in growth regulation (*7*); some of the microsomal H_{1C} sites are associated with cytochromes P450 (*8*). Tamoxifen, amitriptyline, and fluoxetine also bind potently to H_{1C} (*1,6*); like DPPE, they inhibit mitogenesis in vitro and significantly enhance the growth of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in vivo (*1,9*); we (*5*) have postulated that potency to bind H_{1C} may correlate with tumor growth-promoting activity.

A number of other drugs (*5*), including H_1-antihistamines commonly prescribed

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See "Notes" section following "References."

the treatment of allergic symptoms
:h as seasonal hayfever or sold over-
:-counter for relief of insomnia or cold
mptoms, bind to H_{1C} over a wide range
potencies (*10*). Therefore, we assessed
e following five H_1-antihistamines for
vivo tumor growth stimulation: 1) lora-
dine (Claritin, Schering-Plough Corp.,
enilworth, N.J.), a nonsedating tricyclic
gent structurally similar to amitriptyline;
) astemizole (Hismanal, Janssen Phar-
aceutica, Piscataway, N.J.), nonsedat-
g; 3) cetirizine (Reactine, Pfizer
onsumer Health Care, Division of Pfizer
c., Parsippany, N.J.), sedating; 4) hy-
roxyzine (Atarax, Roerig, Division of
fizer Pharmaceuticals, New York,
.Y.), sedating; and 5) doxylamine (Uni-
om, Pfizer Consumer Health Care; Vicks
NyQuil, Richardson-Vicks Inc., Cincin-
ati, Ohio), sedating.

Reliable in vitro correlative assays could be useful in the preclinical screening of drugs for tumor growth promotion. On the basis of earlier indications, a major aim of this investigation was to evaluate the predictiveness of five in vitro tests. Previously, we reported a correlation among potencies of DPPE and certain H_1- and H_2-antihistamines to compete at H_{1C} sites in rat liver microsomes and to inhibit DNA synthesis in concanavalin A-stimulated mouse spleen cells (*10*) and between potencies of fluoxetine and amitriptyline to stimulate DNA synthesis in C-3 fibrosarcoma cells with a bell-shaped dose–response curve, to inhibit mitogenesis, and to bind to nuclear H_{1C} (*1*). In addition, the demonstration in adrenal microsomes that some H_{1C} sites represent P450 (*8*) suggested that the activity of this family of monooxygenases may be implicated not only in carcinogenesis (*11*), but also in tumor growth promotion.

Therefore, we assessed the potency of each antihistamine in the following in vitro assays: 1) inhibition of [^{3}H]histamine binding to microsomal H_{1C}, 2) inhibition of histamine binding to microsomal P450, 3) inhibition of the P450-catalyzed demethylation of aminopyrine, 4) inhibition of lymphocyte mitogenesis, and 5) stimulation of tumor colony formation. For each agent, the overall cumulative score in the battery of tests was correlated with propensity, at estimated human-equivalent doses, to stimulate the in vivo growth of two transplantable murine tumors (B16F10 melanoma and C-3 fibrosarcoma).

Materials and Methods

In Vitro Assays

Inhibition of [^{3}H]histamine binding to H_{1C} in rat liver microsomes. Microsomal fractions were freshly prepared from livers of adult Sprague–Dawley rats, as described previously (6). [^{3}H]Histamine-binding assays (0.5 mg microsomal protein per milliliter) were performed in a buffer of 10 *mM* HCO_3 containing 0.1 *μM* $CuCl_2$. [^{3}H]Histamine (5 nmol; Du Pont NEN Research Products, Boston, Mass.) was incubated at room temperature in the dark for 60 minutes with increasing concentrations of cold histamine or of the test agents. The reaction was terminated by centrifugation at 12 000*g* for 15 minutes at 4 °C. Radioactivity was quantitated for replicate samples, and binding data were analyzed using the LIGAND program (*12*) (three to four tests). Under the binding conditions employed, microsomes were found to contain two sites for histamine (K_{d1} [dissociation constant for high-affinity site] = 0.26 ± 0.19 *μM* [mean ± SEM]; K_{d2} [dissociation constant for low-affinity site] = 36 ± 15 *μM*).

Inhibition of histamine binding to microsomal P450. The K_i (inhibitory constant) value of each antihistamine for histamine binding to P450 in rat liver microsomes was determined by analyses of difference spectra. A Milton Roy Spectronic 3000 Array Spectrophotometer (Milton Roy Company, New Rochelle, N.Y.) with a computer software-controlled program (Rapidscan) collected and plotted the spectral data. Microsomes were kept frozen (−70 °C) until used, thawed, diluted in potassium phosphate buffer at 4 °C and pH 8.5 (pH optimum for histamine difference spectra), gently homogenized, and preincubated for 30 minutes at 22 °C. Final concentrations of histamine (0.125–1.0 *mM*) and inhibitory doses of antihistamine (20–5000 *μM*) or buffer (100 μL each) were added to rat liver microsomes (1 mg/mL) in 12 × 75-mm polypropylene tubes (final vol = 1 mL) and incubated for 25 minutes at 22 °C prior to readings. Tissue with or without competitors served as references. The ΔA (amount bound), characterized by the difference between a broad trough at 390–410 nm and a peak at 425–435 nm (*13*), was plotted against the histamine concentrations added in the presence or absence of competitors. K_m (Michaelis–Menten constant), and K_i values were determined from Lineweaver–Burke plots analyzed with the IBM-PC program ENZYME (*14*). Typical mean K_m values ± SEM for histamine binding were 364 ± 22 *μM* (three to four tests).

Aminopyrine demethylase assay. Rat liver microsomes were prepared by the method of Boobis et al. (*15*). Eppendorf centrifuge tubes (1.5 mL, capped; Brinkmann Instruments, Inc., Westbury, N.Y.) containing a final concentration of 10 μL of aminopyrine (0.1–2.5 *mM*), 50 μL of regenerating system (glucose-6-phosphate, 5 *mM*; glucose-6-phosphate dehydrogenase, 1 U/mL; and nicotinamide-adenine dinucleotide phosphate, 0.3 *mM*), and 940 μL of microsomes (0.5 mg/protein) were incubated for 20 minutes at 37 °C in 50 *mM* Tris buffer (pH 7.4), containing 5 *mM* $MgCl_2$, 1 *mM* EDTA, and 8.0 *mM* nicotinamide. The microsomal suspension was preincubated in 10-mL, 16 × 120-mm conical glass tubes for 15–20 minutes at 20 °C, with or without test agent, and then mixed with substrate and regenerating system. The antihistamines were dissolved in H_2O or ethanol; the ethanol had no effect on enzyme activity. The reaction was stopped with 0.3 mL of 20% trichloroacetic acid. The tubes were centrifuged at 10 000*g* for 10 minutes at 4 °C, and 0.5 mL of supernatant was added to 0.5 mL of NASH reagent (*16*). The mixture was incubated for 10 minutes at 70 °C, then cooled to room temperature. The product (formaldehyde) was determined by absorbance, measured at 412 nm, in a Milton Roy Spectronic 3000 Array Spectrophotometer. Formaldehyde standards (0–0.2 *mM*) in 1.0 mL of buffer and 0.3 mL of 20% trichloroacetic acid were reacted, and the absorbance was determined. Tubes containing no substrate, with or without test agent, were used as references. Typical Michaelis–Menten values were K_m = 0.4 *mM* and V_{max} (maximal velocity) = 4.8 nmol/mg protein per minute. Lineweaver–Burke plots, K_m and K_i were determined with the IBM-PC program ENZYME by a weighted nonlinear least-squares curve procedure (*17*).

Mitogenesis studies. Fresh spleen cells (5 × 10^5) obtained from 5-week-old BALB/c mice (Charles River, St. Constant, Quebec, Canada) were suspended in RPMI-1640 medium containing 2% fetal calf serum (FCS) (GIBCO BRL, Grand Island, N.Y.), seeded into replicate microwell plates (Nunc, Roskilde, Denmark) to which was added concanavalin A (2.5 μg/mL; Sigma Chemical Co., St. Louis, Mo.), and incubated (37 °C, 95% air, 5% CO_2) in the absence or presence of increasing concentrations of the test agents. Forty-three hours after the addition of concanavalin A, 0.25 nmol [^{3}H]thymidine (6.7 Ci/mmol; ICN Radiopharmaceuticals, Montreal, Quebec) was added to each well. After an additional 5-hour incubation, the cells were washed from the wells onto fiber papers with the use of an automated cell sorter. The filters were placed in vials containing 5 mL of scintillation fluid (Readysafe; Beckman Instruments, Inc., Fullerton, Calif.), and radioactivity incorporated into DNA at 48 hours was determined (three assays for each ligand).

Tumor colony growth assay. To assess the effect of the various antihistamines on the growth of B16F10 melanoma cells in vitro, we used suboptimal culture conditions, employing stale (conditioned) medium as described by Vichi and Tritton (*18*) for doxorubicin. B16F10 melanoma cells (1 × 10^5) in 1 mL of conditioned α-minimal essential medium (α-MEM; GIBCO BRL) containing 10% FCS were added to replicate 10-cm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) containing 9 mL of α-MEM (10% FCS) and 0.1 mL of drug or vehicle solution (final drug concentration: $10^{-4.5}$–10^{-7} *M*). After incubation for 24 hours (37 °C, 95% air, 5% CO_2), the cells from each dish were recovered by combining the supernatants from the original solution, the washed solution, and the trypsinizing solution, followed by centrifugation for 10 minutes (700*g* at room temperature). Each cell pellet was resuspended in 10 mL of fresh α-MEM (10% FCS), diluted 1000-fold (final concentration, approximately 10^2 cells/mL), and 1 mL of the cell suspension

Table 1. Potency of five H_1-antihistamines in five in vitro assays*

	[^{3}H]Histamine binding to H_{1C}			Histamine binding to P450		Aminopyrine demethylase		Mitogenesis		Tumor colony growth		
H_1-antihistamine	K_{i1}, μM*	K_{i2}, μM*	Rank†	K_i, μM*	Rank†	IC_{50}, μM*	Rank†	IC_{50}, μM*	Rank†	% stimulation	Rank†	Score†
Loratadine	2 ± 1	381 ± 114	2	13.5 ± 1.5	1	3.1 ± 0.3	1	1.0 ± 0.5	1	21	3	22
Astemizole	2.8 ± 2.2	36 ± 5.5	1	31 ± 1.7	2	27 ± 2	2	2.0 ± 0.2	2	35	1	22
Hydroxyzine	1.2 ± 0.4	2080 ± 825	3	62 ± 5.6	3	38 ± 8	3	12 ± 1	3	30	2	16
Doxylamine	0.8 ± 0.3	>3500	4	142 ± 35	4	73 ± 8	4	70 ± 5	4	9	4	10
Cetirizine	NB	NB	5	1537 ± 186	5	762 ± 85	5	160 ± 10	5	9	4	6

*Values = means ± SEM (three to six assays for each of the five tests). K_{i1} = inhibitory constant for high-affinity site; K_{i2} = inhibitory constant for low-affinity site; IC_{50} = concentration that causes 50% inhibition; NB = no binding detected.

†Scoring in each assay by rank as follows: rank 1 = 5 points; 2 = 4 points; 3 = 3 points; 4 = 2 points; 5 = 1 point.

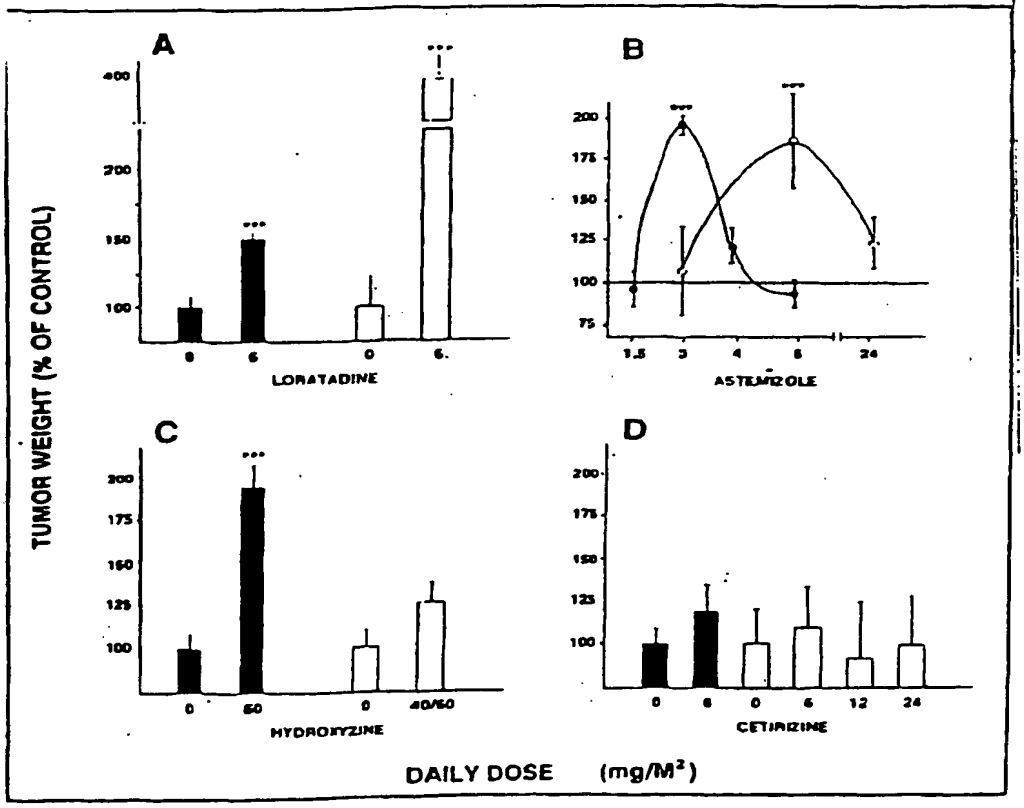

Fig. 1. Effect of loratadine (A), astemizole (B), hydroxyzine (C), and cetirizine (D) on the growth of B16F10 melanoma (■ = A, C, and D; ● = B) and C-3 fibrosarcoma (□ = A, C, and D; ○ = B). ***P<.001; for loratadine, number of mice = 10-11; for hydroxyzine, number of mice = 19-22; for cetirizine, number of mice = 10-21 for each concentration; for astemizole, number of mice = 10 for each concentration (B16F10 melanoma) and number of mice = 20 for each concentration (C-3 fibrosarcoma). Bars = SEM.

duction of various cytochromes P450 and of histidine decarboxylase, the histamine-forming enzyme, is associated with both tumor growth and the mitogenic response (*10*,*25*). A comprehensive review (*26*) of many studies concluded that drug-induced modification of tumorigenesis is associated with the altered expression of a number of enzymes, including cytochromes P450.

That the concentrations of the antihistamines to modulate growth were lower than those required to inhibit histamine binding to P450 may signify a more potent interaction with specific cell P450s that control proliferation and/or are induced in proliferating lymphocytes (*25*) and in malignant cells (*27*), whereas the binding potency in liver microsomes might reflect overall affinity for a composite of P450 enzymes. Similarly, the rank order of potency to inhibit [^{3}H]histamine binding to microsomal H_{1C} at low, but not high, affinity sites (Table 1) correlated with the rank order of potency in the proliferative assays, suggesting that the lower affinity sites represent, at least in part, binding to P450. The observation of a reasonable correspondence between the absolute concentrations of drugs to inhibit aminopyrine demethylase and those to inhibit mitogenesis suggests that the P450 isoenzymes that metabolize aminopyrine are relatively closely linked to mitogenic processes.

As a class, drugs that promote tumor growth tend to be immunosuppressive (*26*,*28*); likewise, the potency of the five antihistamines to inhibit lymphocyte mitogenesis correlated highly with their propensity to stimulate tumor growth in vivo. Thus, this simple and highly reproducible test, requiring little in the way of sophisticated laboratory equipment, may be the easiest assay with which to screen potential tumor growth promoters. For example, DPPE, fluoxetine, and amitriptyline are potent inhibitors of mitogenesis (IC_{50} = 0.1 μM, 0.75 μM, and 2.5 μM, respectively) and accelerate tumor growth in vivo (*1*,*5*). Similarly, the same correlation shown now for astemizole, loratadine, and hydroxyzine raises concerns about other antihistamines that potently (IC_{50}, ≤10 μM) inhibit mitogenesis, including prochlorperazine (Compazine, Smith-Kline Beecham, Philadelphia, Pa.; IC_{50} = 1 μM), promethazine (Phenergan, Wyeth-Ayerst Laboratories, Philadelphia, Pa.; IC_{50} = 5 μM), and terfenadine[2] (Seldane, Marion Merrell Dow Inc.; IC_{50} = 10 μM).

We recognize that the predictor tests have been applied to a small number of

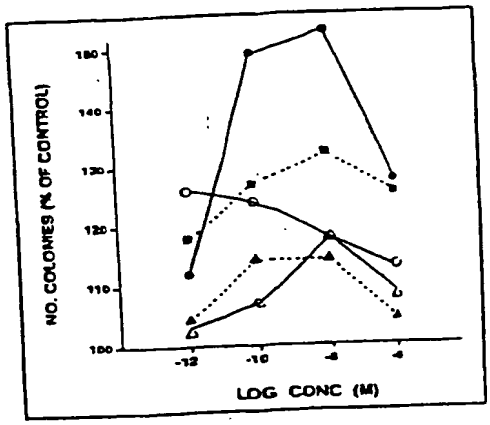

Fig. 2. Stimulation of B16F10 melanoma colony growth in vitro by loratadine (○), astemizole (●), hydroxyzine (■), doxylamine (▲), and cetirizine (△), as described in "Materials and Methods" section.

Table 2. Correlation between rank order of potency of antihistamines in five in vitro assays and rank order to promote tumors in vivo*

In vitro assay	r†
[^{3}H]Histamine binding	.95‡
Histamine binding to P450	.95‡
Aminopyrine demethylase	.95‡
Mitogenesis	.81
Tumor colony growth	.97‡
Cumulative score—five assays	.97‡

*Potency to promote tumors in vivo was ranked according to whether the drug significantly stimulated the growth of C-3 fibrosarcoma and B16F10 melanoma. Based on this criterion, loratadine and astemizole were each ranked first (significant promotion of both tumors); hydroxyzine ranked third behind loratadine and astemizole (significant promotion of B16 melanoma), and doxylamine and cetirizine each ranked fourth (no significant promotion of either tumor). Thus, rank order in each in vitro assay and rank order of cumulative scores in all five assays (Table 1) were compared with the rank order 1, 1, 3, 4, and 4 in the in vivo tumor promotion assays.

†Spearman's coefficient of rank correlation.

‡$P<.02$.

compounds. Moreover, drug administration was by the intraperitoneal route only and may have yielded results different from those obtained with oral administration, the latter characterized by variable absorption and first-pass liver metabolism.[3] Also, as exemplified by the bell-shaped dose–response curves and the differing effects of astemizole and hydroxyzine on the growth of B16F10 melanoma and C-3 fibrosarcoma, tumor promotion may depend on both the tumor type and the drug dose; a panel of several transplantable tumor lines or additional models, such as 7,12-dimethylbenz[*a*]anthracene-induced carcinogenesis (*1,2,5*) or human tumors implanted into nude mice, might best expose the propensity of drugs, administered over a wide human-equivalent dose range, to stimulate tumor growth.

Although the potential for carcinogenicity has received considerable attention in preclinical drug testing in rodents, the propensity of pharmaceuticals to enhance the growth of existing tumors or the development of malignancy induced by chemical or viral initiators has been neglected. While caution must be exercised in extrapolating our data from rodents to humans, we believe that epidemiologic studies (*29*) may further contribute to an understanding of the potential risks that our laboratory findings have exposed.

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for this study. Personal interviews solicited information on age, income, marital status, place of birth, education, health insurance coverage, Pap smear- and mammogram-screening practices, and six questions relating to social network. A social network score was assigned to each woman by sum-

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See "Notes" section following "References."